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Effect of Glutamine-Supplemented Extender on Kinematics and Quality of Cryopreserved Epididymal Sperm of Barb Stallions

Nedjma Aouane[1], Ahmed Khireddine Metref[2*], Ibrahim Nabi[3] and Redha Belala[2]

[1]Higher National Veterinary School of Algiers, Algeria
[2]Institute of Veterinary Sciences, University of Blida, Algeria
[3]Department of Natural and Life Sciences, Dr. Yahia Fares University, Médéa, Algeria

ABSTRACT

The aim of the present study was to evaluate the effect of glutamine on the motility parameters after thawing of epididymal semen of Barb stallions. Semen samples were collected from 22 testicles of slaughtered Barb stallions by retrograde flushing technique. Semen samples were aliquoted and diluted with the following extenders, (1) base medium (BM) (INRA96®+2.5%glycerol+2% egg yolk); (2) BM+25mM Glutamine (C25); (3) BM+50mM Glutamine (C50); (4) BM+75mM Glutamine (C75) and (5) BM+100mM Glutamine (C100). The assessment of Kinematic parameters was established after thawing by using SPA® spain CASA (Computer Automated Sperm Analyzer); and the plasma membrane functional integrity was analyzed by HOST test. At low concentrations, the presence of glutamine (C25 and C50) improved significantly ($p<0.05$) the kinematics variables (VSL, VAP, LIN and STR). However, higher concentrations of glutamine (C75 and especially C100) significantly decreased the movement of thawed semen. In conclusion, these observations are showing a beneficial effect of glutamine on sperm kinematics at low concentrations and indicated a toxic effect at high concentrations, although, no differences were observed in terms of HOST integrity.

Keywords: Glutamine, Epididymal sperm, Cryopreservation, Barb stallions, CASA

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[*]Corresponding author.
E-mail address: metref_ahmed@univ-blida.dz (Ahmed Khireddine Metref)
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INTRODUCTION

The Barb horse is a national emblem in Arabian Maghreb, and occupies an important place in the history, culture and the traditions of the society. It is a versatile and enduring horse that adapts easily to different climates both in the countries where the breed originated (Morocco, Algeria, and Tunisia) and in the countries where it has been exported from Europe to sub-Saharan Africa. It is generally used in Fantasia (traditional equestrian exhibition in the Maghreb) as well as in equestrian sports (endurance and show jumping) (Rahal, 2005; Rahal *et al*., 2009 and Berber *et al*., 2014). Their exceptional faculties of learning, assimilation and understanding (Chabchoub *et al*., 2004), represents an attracting interest in riding clubs and endurance races internationally (Rahal, 2005; Rahal *et al*., 2009; Berber *et al*., 2014).Despite, the majority of the studies on Barb focused on morphometric parameters, few reports were interested in the reproduction and preservation of this breed. In a prime study on fertility parameters, Aouane et al (2019) showed that the fertility of the Barb was better than other breeds studied in natural mating, regardless of age; and showed that, when the age of Barb stallion increases, the conception rate per cycle increases too, within a significant interaction. These observations impose the needs to exploit and preserve the high fertility of older Barb stallions, which unfortunately, oriented to be reformed or slaughtered because of their ages or loco-motor injuries occurred during a long span of an equestrian life. Therefore, the use of epididymal sperm is a valuable mean to preserve high quality stallions after reform, castration, serious injury, acute illness or sudden death. Currently, stallion epididymal sperm diluted with an appropriate extender can be stored successfully under cooled conditions for up to 24-72 h (Guimarãesa *et al*., 2012; Monteiro *et al*., 2013 and Vieira *et al*., 2013); and many studies reported several works on cryopreserved epididymal sperm with variable pregnancy rates (27-66%) (Papa *et al*., 2008 and Heise *et al*., 2010). The physiological response of epididymal sperm subjected to cryopreservation is a topic of interest, and cannot be discussed similarly to ejaculated semen; while the harvesting method is different and the seminal plasma is absent. In general, the low fertility of frozen equine semen (Amann and Pickett, 1987) is partly caused by the complex process of sperm freezing and thawing which causes several types of cellular lesions (likewise the disruption of phospholipids organization and hyperosmotic stress; Hammerstedt *et al*., 1990 and Garner, 1991), these lesions can be mitigated by the use of glycerol as a conventional cryoprotectant and commonly used in the freezing medium, which likely has an obvious toxicity (Demick *et al*., 1976 and Jasko *et al*., 1992). In order to improve the characteristics of stallion semen, the use of amino acids is having beneficial interests. Several reports showed that amino acids have been protective in several organisms and animal cells in response to cold temperatures (Anchordoguy *et al*., 1988; Heinz *et al*., 1990; Kruuv and Glofcheski, 1990; Lalonde *et al*., 1991). In spermatozoa, it has been demonstrated that the addition of taurine to chilled semen of cats (Baran *et al*., 2009), rams (Bucak *et al*., 2007) and stallions (Ijaz and Ducharme, 1995) improved the motility. The protective effect of both glutamine (Trimeche *et al*., 1996a, 1999 and Khlifaoui *et al*., 2005) and proline (Trimeche *et al*., 1999) against freezing–thawing damage in stallion and donkey spermatozoa have also been reported.

In Algeria, many difficulties are involved in the limited use of equine artificial insemination or the use of ejaculated/epididymal stallion sperm cryopreservation. For the knowledge of the authors, no previous reports investigated the cryopreservation the Barb sperm. The aim of this study was to evaluate the motility parameters of cryopreserved epididymal sperm of Barb stallions after incorporation of glutamine in freezing extender.

MATERIALS AND METHODS

Ethical approval: The research was approved by the Research Ethics Committee of our university

Animal and epididymal sperm collection: Testicles of mature stallions of local Barb breed were immediately collected after slaughter at the El Harrach slaughterhouse (Algiers). The gonads were transported to the Laboratory of Biotechnology of Animal Reproduction of the University of Blida (LBRA) in a chilled-tank within 2 hours after slaughtering. After dissection of cauda epididymis, the organ was washed with physiological saline solution and dried, and a 21G needle was attached to a 5 mL air-filled syringe inserted into the vas deferens. The sperm was collected by retrograde flushing from the vas deferens through the tail of the epididymis into a 1.5 mL Eppendorf® tube (Vieira et al, 2013). The epididymal sperm volume and concentration were measured for the motility without HOST (Table 2). The remaining epididymal fluid was used for treatment and cryopreservation.

Semen samples, dilution and freezing procedure: Only samples containing concentration of > 10x 10^9 spz/ml and a total motility > 60%, were split and assigned to one of the five treatments (Table 1). The semen-diluter-treatment mixture was gradually cooled from 37°C to + 4°C for 1 hour in the refrigerator (equilibration). It was packaged in 0.5 ml straws at 4°C and frozen in nitrogen vapors for 10 minutes at 4 cm. The straws were then immersed in liquid nitrogen at (-196°C) (Heise *et al*., 2010 and Moreno *et al*., 2013).

Table 1: Composition of freezing media tested

Freezing medium	Composition
Basic Medium (MB)	INRA 96®+ (2% EY or 4% EYP) + 2.5% Glycerol
Glutamine	
C25	MB+ 25 mM Glutamine
C50	MB+ 50 mM Glutamine
C75	MB+ 75 mM Glutamine
C100	MB+ 100 mM Glutamine

INRA 96® (200 ml, IMV -Technologies, L'aigle, France); Glutamine (Sigma-Aldrich®, Germany); Glycerol (Sigma-Aldrich®, Germany)

Motility and membrane integrity analysis: The frozen semen was thawed by immersing the straws in a water bath at 37°C for 30 seconds. The contents were then transferred to pre-warmed 1.5 mL Eppendorf® tubes (37°C) and analyzed for sperm motility and membrane integrity. Semen was analyzed using SPA® spain CASA analyser, to determine total motility, mobile spermatozoa trajectory and velocities like curvilinear velocity (VCL), progressive velocity (VSL), linearity (LIN= VSL/VCL x 100), the lateral amplitude of the head beat (ALH), the average progressive velocity (VAP) and the progressive motility (number of undulating progressive spermatozoa/total number of motile spermatozoa) by incubating the sperm at 37°C for 10 minutes (Heise et al, 2010; Monteiro et al, 2013, Moreno et al, 2013)

The HOST (hypo-osmotic swelling test) assesses the functional integrity of the spermatozoa plasma membrane and also serves as a useful indicator of sperm fertility potential (Talluri *et al*., 2017). The hypo-osmotic solution consisted of 0.735 g sodium citrate, 1.351 g fructose and 100 mL distilled water. The osmolality of the solution was 150 mOsm/kg and the pH was 7.9. Aliquots (0.1 mL) of the semen samples were mixed with 1mL of the pre-warmed hypo-osmotic solution and incubated at 37°C for 1 h. After the incubation period, the smears were prepared using 5 µl aliquots. One hundred spermatozoa were examined with a phase contrast microscope for the percentage of changes in the tail of the sperm typical of the hypo-osmotic swelling test (Trimeche *et al*., 1999)

Statistical analysis: The statistical analysis of the results was performed using the STATVIEW software (Version 5.0) for Windows TM. The values analyzed by ANOVA were expressed in mean ± Standard Deviation. Differences in mobility and membrane integrity values were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The mobility and movement under the glutamine effect (characteristics of thawed semen)

Table 2 shows the motility parameters of thawed epididymal sperm of Barb stallions after being subjected to different glutamine concentrations. Results showed a decrease in the percentages of total motility (MOT) and progressive motility (PMOT) after thawing in comparison to values of fresh semen (79.28±2.12% and 42.36±2.38% vs 22.2.±4.37% and 7.12±2.59%, respectively).

When comparing the motility of glutamine treated samples to the control (Base medium), a decrease in MOT and PMOT was observed in all treatments; however, statistical analysis revealed no significant effect. The effect of glutamine treatments on kinematic parameters (VCL, VAP and VSL) was dependent on the glutamine concentration. As shown in Table 2, glutamine C25 and C50 significantly ($p<0.05$) improved VAP (19.01; 19.26 µm/s vs 17.1 µm/s) and VSL (14.14; 14.51 µm/s vs 11.93 µm/s), respectively. On the other hand, greater concentrations glutamine (C75 and C100) decreased significantly ($p<0.05$) values of VCL (26.99; 24.4 µm/s vs 29.83) and ALH (1.14; 1.13 µm vs 1.23 µm) compared to control, this explains that, these parameters are important for evaluating sperm quality and the ability of sperm to move effectively and fertilize an egg. In our study the higher concentration of glutamine (C75, C100) decreased these parameters; whereas lower concentration led to increase them. The treatment C100 showed a significant decrease ($p<0.05$) in VCL, VAP and VSL compared to control and all other treatments. Our results showed a significant ($p<0.05$) increase in LIN (linearity) and STR (straightness) when treated with glutamine C50 and C75 compared to control. However, glutamine C100 showed a decrease in LIN and STR compared to the other treatments. Compared to control, glutamine C25 showed a significant ($p<0.05$) increase in the amplitude of lateral head movement (ALH).

The membrane integrity of thawed sperm (figure 1) under the glutamine effect: For the reactivity of spz under different glutamine treatments, we obtained a decrease in cryo-survival of spz in all treatments; this was not statistically demonstrated because the decrease was not statistically significant. In the present study, Glutamine has been supplemented in different concentrations to base freezing medium to improve the post-thaw quality of cryopreserved epididymal Barb semen. Several previous studies showed a positive effect of glutamine to preserve semen in different species; although, we believe in the scale of our knowledge that the frozenness of Barb semen or its response to glutamine treatment has not been studied before

On the basis of our findings the percentages of total motility and progressive motility were not significantly improved for the different concentrations of glutamine used. However, the positive effect of glutamine was observed on kinematic parameters with medium concentrations of 25 and 50 mM of glutamine, because when kinematic param-

Table 2: Effect of glutamine at different concentrations on the kinematic parameters of epididymal sperm of Barb stallions after thawing

Sperm motility characteristics	Fresh semen	Thawed Epididymal semen				
		Control (MB)[1]	Glutamine Supplements			
			C 25	C50	C 75	C100
Motility (%)	78.85±09.88	22.20±4.37^{ns}	16.48±2.60^{ns}	20.94±5.13^{ns}	12.17±2.46^{ns}	11.76±2.20^{ns}
PMOT (%)	42.63±10.52	07.12±2.59^{ns}	03.61±1.08^{ns}	8.03±3.69^{ns}	03.15±1.06^{ns}	2.57±1.15^{ns}
VCL (µm/s)	51.13±08.78	29.83±0.81^{a}	30.40±0.59^{a}	30.04±0.66^{a}	26.99±0.74^{b}	24.40±0.62^{b,c}
VAP (µm/s)	34.67±08.43	17.10±0.54^{a}	19.01±0.45^{b}	19.26±0.51^{b}	17.70±0.58^{a,b,c}	15.15±0.49^{b,c,d}
VSL (µm/s)	28.64±08.32	11.93±0.49^{a}	14.14±0.42^{b}	14.51±0.47^{b}	13.69±0.55^{b}	11.27±0.47^{a}
LIN (%)	78.3±0.09	31.61±0.84^{a}	37.65±0.73^{b,c}	38.04±0.76^{b,c}	39.68±0.95^{b}	36.45±0.95^{b,c}
STR (%)	55.9±0.13	53.89±1.01^{a}	60.01±0.81^{b}	60.65±0.82^{b}	62.34±0.99^{b}	54.79±01.02^{b}
ALH (µm)	1.92±0.36	01.23±0.03^{a}	01.31±0.23^{b}	01.21±0.23^{a}	01.14±0.23^{b,c}	01.13±0.25^{b,c}

Mean values ± S.E.M. of 22 epididymal sperm samples (n=22). Significant difference ($p < 0.05$) indicated by the different letters (a, b, c).

C: L-glutamine concentration; Motility: percentage of motile spermatozoa, PMOT: percentage of spermatozoa with progressive motility; VCL: curvilinear velocity; VAP: mean progressive velocity; linearity LIN: VSL/VCL; STR: VAP/VCL; progressive velocity (VSL), lateral amplitude of headbeat (ALH)

[1]: Base Medium, INRA 96® Medium + 2.5% glycerol and 2% egg yolk or 4% egg yolk plasma.

eters are improved it's expected that mobility parameters would also be improved. However, there are other factors incriminated like: sperm DNA damage, imbalance hormone level, problem in sperm maturation (Muratori et al., 2015). Trimeche et al (1999), Khlifaoui et al (2003) and Khlifaoui et al (2005) reported a significant improvement in post-thaw mobility of stallion sperm following the addition of 50 mM of glutamine. In another study, Trimeche et al (1996a) observed that the presence of 80mM glutamine in the INRA82 diluent modified with 4% (v/v) glycerol and 2% (v/v) quail egg yolk significantly improved the post-thaw motility of Poitou Jackass spermatozoa. For human spermatozoa, Renard et al (1996) reported that the presence of 80 mM glutamine in a freezing diluent in the presence of glycerol improved mobility and fertilization capacity after thawing. At optimized concentration ranging between 100 and 150 mM of L-glutamine, L- proline and L-glycine exhibit proconservation efficacy by maintaining approximately 11- 17 % of total sperm motility in contrast amino acids such as L-arginine, lysine and L-histidine showed cryoprotective effects, preserving only 0-5% sprem motility . In our study, the positive influence of glutamine was significantly observed when analyzing the kinematic parameters of mobility. Thus, glutamine C25 and C50 significantly ($p<0.05$) improved VAP and VSL. Our results are consistent with those of Trimeche et al (1999) for VAP, VSL and VCL (40 and 50 mM glutamine) in stallion semen, as well as those of El-Sheshtawy et al (2008) with the use of 25-50 mM and Ibrahim Mahmoud and Ibrahim Saad (2016) who showed that the addition of glutamine at 20-60 mM to the Tris+Egg Yolk diluent not only improved sperm motility and viability parameters but also decreased sperm abnormalities. At lower concentrations (glutamine and proline 10, 20 mM), the use of amino acids as cryoprotectants have shown improve stallion sperm motility after thawing, but no kinematics parameters were observed (Trimeche *et al*., 1999). While other combinations are more beneficial, Amirat-briand et al (2009) showed the cryoprotective role of glutamine (10 mM) in combination with 6.4% glycerol on bull semen and glutamine at 10 mM + LDL improved semen motility during the thawing freezing process of bovine semen. In Goat semen, the combination of 25 mM glutamine + 8% LDL+ 6.4% glycerol showed a cryoprotective effect (Ali al Ahmed *et al*., 2008). However, in sheep, lower glutamine supplementation (5 mM) had been of greater benefit to frozen- thawed ram semen by improving sperm motility and viability (Bucak *et al*., 2009). Unlikely, Renard et al (1996) concluded that glutamine and proline at 8 or 20 mM had no cryoprotective effect on human semen after freeze-thawing. We noted, in the present study, that the high concentrations of glutamine C75 and C100 significantly decreased VCL and ALH. However, glutamine C100 significantly decreased all movement parameters namely VCL, VAP and VSL in addition to LIN linearity and STR straightness compared to control and also to all other treatments. This decrease could be explained by the reported work on the toxicity of higher amino acid concentrations during the freeze-thaw process. The higher concentration of L-glutamine between 120-160 mM explained the toxicity by deleterious osmotic

effects (Kruuv *et al*., 1988; Sanchez-Pardia *et al*., 1992; Renard *et al*., 1996; Trimeche *et al*., 1996a) and concluded that the protective effect of glutamine is neutralized by the sensitizing effect of hypertonicity. In studies by Ibrahim Mahmoud and Ibrahim Saad (2016) and El-Sheshtawy et al (2008) on bovine semen freezing, they also found that glutamine at 100 mM significantly reduced motility and viability parameters. Consistently to our findings, Khlifaoui et al (2005) showed a decrease in motility after thawing of stallion semen when using 50-100 mM glutamine and 150 mM in the presence of 2.5% glycerol and concluded that glutamine at high concentrations (>0.5%) has a toxic biochemical effect (>100mM). However, the use of 100 mM glutamine in the presence of low concentration of 1.5% glycerol did not affect motility after thawing; suggesting that the toxic effect of glutamine was related to increased osmotic pressure and not to the use of high concentrations of glutamine, because that is the consequence of the use of high concentration of glutamine and glycerol at the same time, increase the osmotic pressure leading to cellular dehydration and oxidative stress, however when we use only low concentration of glycerol , the high concentration of glutamine didn't affect the motility. However, Trimeche et al (1996a) concluded that at higher concentration of glutamine has osmotic toxicity without excluding the hypothesis of biochemical toxicity. In the present study, glutamine C50 and C75 significantly improved linearity (LIN) and straightness (STR), both of which are considered good indicators of progressive spz mobility. However, glutamine C25 significantly increased the amplitude of lateral head displacement (ALH) in contrast to what Trimeche et al (1996b) had found. Regarding membrane integrity, the use of glutamine at different concentrations did not improve the viability of epididymal sperm of the Barb stallions tested in our study (P value < 0,05). Forthemore, Khlifaoui et al (2003) reported, that 50 mM of glutamine improved not only membrane integrity but also DNA integrity and acrosome preservation of cryopreserved stallion semen. Several studies have shown the independence of the cryoprotective mechanism of glycerol and glutamine (synergetic role;Kruuv and Glofcheski, 1990; Renard *et al*., 1996; Trimeche *et al*., 1996a). Khlifaoui et al (2005). Kruuv and Glofcheski (1990) hypothesized that there is a glutamine binding site on a plasma protein. Additional studies must be carried out to determine the exact mechanism of action of glutamine on membrane integrity in light of the hypotheses put forward by Trimeche et al. (1996b).

CONCLUSIONS

The reactivity of the epididymal sperm of the Barb stallion to the addition of glutamine to the freezing medium was positively influenced at low concentrations (C25 and C50) in terms of kinematic movement parameters (VAP, VSL) and especially linearity and straightness which are considered indicators of progressive movement. Further studies are needed to explore the effect of glutamine on Barb's epididymal sperm or to evaluate other treatment combinations.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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